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APPLICATION

FOR

UNITED STATES LETTERS PATENT

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TITLE : ANTISENSE IAP OLIGONUCLEOTIDES AND USES THEREOF

ANTISENSE IAP OLIGONUCLEOTIDES AND USES THEREOF

Cross-Reference to Related Applications

This application is a continuation of U.S.S.N. 09/672,717 (now allowed), filed September 28, 2000.

Field of the Invention

The invention relates to antisense IAP oligonucleotides and methods of using them to increase apoptosis.

Background of the Invention

One way by which cells die is referred to as apoptosis, or programmed cell death. Apoptosis often occurs as a normal part of the development and maintenance of healthy tissues. The process may occur so rapidly that it is difficult to detect.

The apoptosis pathway is now known to play a critical role in embryonic development, viral pathogenesis, cancer, autoimmune disorders, and neurodegenerative diseases, as well as other events. The failure of an apoptotic response has been implicated in the development of cancer, autoimmune disorders, such as lupus erythematosis and multiple sclerosis, and in viral infections, including those associated with herpes virus, poxvirus, and adenovirus.

Baculoviruses encode proteins that are termed inhibitors of apoptosis (IAPs) because they inhibit the apoptosis that would otherwise occur when insect cells are infected by the virus. These proteins are thought to work in a manner that is independent of other viral proteins. The baculovirus IAP genes include sequences encoding a ring zinc finger-like motif (RZF), which is presumed to be directly involved in DNA binding,

and two N-terminal domains that consist of a 70 amino acid repeat motif termed a BIR domain (Baculovirus IAP Repeat).

The role of apoptosis in cancer has only recently been appreciated. The identification of growth promoting "oncogenes" in the late 1970's gave rise to an almost universal focus on cellular proliferation that dominated research in cancer biology for many years. Long-standing dogma held that anti-cancer therapies preferentially targeted rapidly dividing cancer cells relative to "normal" cells. This explanation was not entirely satisfactory, since some slow growing tumors are easily treated, while many rapidly dividing tumor types are extremely resistant to anti-cancer therapies. Progress in the cancer field has now led to a new paradigm in cancer biology wherein neoplasia is viewed as a failure to execute normal pathways of programmed cell death. Normal cells receive continuous feedback from their neighbors through various growth factors, and commit "suicide" if removed from this context. Cancer cells somehow ignore these commands and continue inappropriate proliferation. Cancer therapies, including radiation and many chemotherapies, have traditionally been viewed as causing overwhelming cellular injury. New evidence suggests that cancer therapies actually work by triggering apoptosis.

Both normal cell types and cancer cell types display a wide range of susceptibility to apoptotic triggers, although the determinants of this resistance are only now under investigation. Many normal cell types undergo temporary growth arrest in response to a sub-lethal dose of radiation or cytotoxic chemical, while cancer cells in the vicinity undergo apoptosis. This provides the crucial treatment "window" of appropriate toxicity that allows successful anti-cancer therapy. It is therefore not surprising that resistance of tumor cells to apoptosis is emerging as a major category of cancer treatment failure.

Compared to the numerous growth-promoting oncogenes identified to date (>100), relatively few genes have been isolated that regulate apoptosis. The Bcl-2 gene was first identified as an oncogene associated with the development of follicular lymphomas. In

contrast to all other oncogenes identified to date, Bcl-2 displays no ability to promote cell proliferation, and instead has been demonstrated to suppress apoptosis by a variety of triggers. Elevated Bcl-2 expression is associated with a poor prognosis in neuroblastoma, prostate and colon cancer, and can result in a multidrug resistant phenotype *in vitro*. Although the study of Bcl-2 has helped revolutionize cancer paradigms, the vast majority of human malignancies do not demonstrate aberrant Bcl-2 expression.

In contrast to the findings with Bcl-2, mutation of the p53 tumor suppresser gene has been estimated to occur in up to 50% of human cancers and is the most frequent genetic change associated with cancer to date. The p53 protein plays a crucial role in surveying the genome for DNA damage. The cell type and degree of damage determines whether the cell will undergo growth arrest and repair, or initiate apoptosis. Mutations in p53 interfere with this activity, rendering the cell resistant to apoptosis by a wide range of cellular insults. Some progress has been made in understanding the molecular biology of p53, but many questions remain. p53 is known to function as a transcription factor, with the ability to positively or negatively regulate the expression of a variety of genes involved in cell cycle control, DNA repair, and apoptosis (including the anti-apoptotic Bcl-2 gene described above and the related pro-apoptotic gene Bax). The drug resistant phenotype conferred by p53 alterations has been linked to Bcl-2/Bax regulation, but this correlation does not hold for most cancer types, leaving open the possibility that other critical genes regulated by p53 remain to be identified.

Summary of the Invention

We have discovered that inhibitor of apoptosis (IAP) protein overexpression is associated with a wide range of cancer types including ovarian cancer, adenocarcinoma, lymphoma, and pancreatic cancer. In addition, we have found that nuclear localization, fragmentation of the IAPs, and overexpression of the IAPs in the presence of p53 mutations correlate with a cancer diagnosis, a poor prognosis, and resistance to numerous

chemotherapeutic cancer drugs. These discoveries provide diagnostic, prognostic, and therapeutic compounds and methods for the detection and treatment of proliferative diseases. One way in which the expression of an IAP in a cell can be decreased is by administering to the cell a negative regulator of the IAP apoptotic pathway, for example, an antisense nucleic acid.

In general, the invention features methods and reagents useful for inducing apoptosis in a cell. The methods and reagents of the invention are useful in treating cancers, and other proliferative diseases.

In a first aspect, the invention features an inhibitor of apoptosis (IAP) antisense oligonucleotide that inhibits IAP biological activity, regardless of the length of the antisense oligonucleotide. In preferred embodiments, the IAP is XIAP, HIAP1, or HIAP2. In other preferred embodiments, the antisense oligonucleotide is mammalian, for example, mouse or human. In yet another embodiment, the antisense oligonucleotide is between 8 and 30 nucleotides in length.

In still other further preferred embodiments, the XIAP antisense is chosen from any one of SEQ ID NOS: 1 through 96, and the HIAP1 antisense is chosen from any one of SEQ ID NOS: 97 through 194. Preferably the IAP biological activity is inhibition of apoptosis or inhibition of IAP RNA or polypeptide expression. The antisense oligonucleotide may comprise at least one modified internucleoside linkage. Preferably the modified internucleoside linkage is a phosphorothioate, a methylphosphonate, a phosphotriester, a phosphorodithioate, or a phosphoselenate linkage. In addition, the antisense nucleic acid may comprise at least one modified sugar moiety. Preferably this modified sugar moiety is a 2'-O methoxyethyl group or a 2'-O methyl group. In still another preferred embodiment, the antisense oligonucleotide is a chimeric nucleic acid. Preferably the chimeric oligonucleotide comprises DNA residues linked together by phosphorothioate linkages, and the DNA residues are flanked on each side by at least one 2'-O methyl RNA residue linked together by a phosphorothioate linkage. More

preferably the DNA residues are flanked on each side by at least three 2'-O methyl RNA residues. In yet another embodiment, the antisense oligonucleotide is a ribozyme.

In a second aspect, the invention features a method of enhancing apoptosis in a cell by administering to the cell a negative regulator of the IAP-dependent antiapoptotic pathway. In preferred embodiments the negative regulator is an antisense IAP oligonucleotide, an antibody that specifically binds an IAP polypeptide, an IAP polypeptide comprising a ring zinc finger, the polypeptide having no more than two BIR domains, a nucleic acid encoding the ring zinc finger domain of an IAP polypeptide, or a compound that prevents cleavage of the IAP polypeptide.

In preferred embodiments of the second aspect of the invention, the cell is in a mammal diagnosed with a proliferative disease, for example, cancer. The cell may comprise a mucosa-associated lymphoid tissue (MALT), a tissue in which the IAP gene HIAP1 is frequently involved in a translocation, resulting in marginal zone cell lymphomas. The cell may also be a breast cancer cell, where increased HIAP1 expression is known to correlate with tumor progression. The cell may also be a cell in which NFkB expression or activity is increased, for example, cell of head and neck carcinomas, adult T-cell lymphomas, nasopharyngeal carcinomas, and Hodgkin's disease. The cell may also be an acute myelogenous leukemia cell, where increased XIAP levels correlate with poor patient prognosis. In addition, the cell may be a small cell lung carcinoma cell, where increased levels of XIAP correlates with increased resistance to radiation treatment.

In preferred embodiments of the second aspect of the invention, the IAP is XIAP, HIAP1, or HIAP2. Preferably the antisense oligonucleotide is mammalian, for example, mouse or human. In still other preferred embodiments, the XIAP antisense oligonucleotide is chosen from any one of SEQ ID NOS: 1 through 96, and the HIAP1 antisense oligonucleotide is chosen from any one of SEQ ID NOS: 97 through 194.

In still other embodiments of the second aspect of the invention, the antisense oligonucleotide comprises at least one modified internucleoside linkage. Preferably the modified internucleoside linkage is a phosphorothioate, a methylphosphonate, a phosphotriester, a phosphorodithioate, or a phosphoselenate linkage. In addition, the antisense oligonucleotide may comprise at least one modified sugar moiety. Preferably this modified sugar moiety is a 2'-O methoxyethyl group or a 2'-O methyl group. In still another preferred embodiment, the antisense oligonucleotide is a chimeric nucleic acid. Preferably the chimeric oligonucleotide comprises DNA residues linked together by phosphorothioate linkages, and the DNA residues are flanked on each side by at least one 2'-O methyl RNA residue linked together by a phosphorothioate linkage. More preferably the DNA residues are flanked on each side by at least three 2'-O methyl RNA residues. In still further embodiments, administration of the antisense oligonucleotide sensitizes the cell to chemotherapy or radiotherapy. In addition, the cell may be *in vitro* or *in vivo*.

In a third aspect, the invention features a pharmaceutical composition comprising a mammalian IAP antisense oligonucleotide. In one preferred embodiment, the mammalian antisense IAP oligonucleotide is a human antisense nucleic acid. Preferably the antisense oligonucleotide binds a target sequence of the human XIAP gene or mRNA, the human HIAP1 gene or mRNA, the human HIAP2 gene or mRNA, the murine XIAP gene or mRNA, the murine HIAP1 gene or mRNA, or the murine HIAP2 gene or mRNA. More preferably the composition comprises an antisense oligonucleotide chosen from any one of SEQ ID NOS: 1 through 96 (XIAP) or SEQ ID NOS: 97 through 194 (HIAP1).

In another aspect, the invention features an IAP gene nucleic acid fragment or antisense RNA sequence for use in suppressing cell proliferation. Such nucleic acids of the invention and methods for using them may be identified according to a method involving: (a) providing a cell sample; (b) introducing by transformation into the cell sample a candidate IAP nucleic acid; (c) expressing the candidate IAP nucleic acid within

the cell sample; and (d) determining whether the cell sample exhibits an altered apoptotic response, whereby decreased apoptosis identifies an anti-proliferative compound. Preferably the cell is a cancer cell.

In another aspect, the invention features a method of treating a patient diagnosed with a proliferative disease. In the method, apoptosis may be induced in a cell to control a proliferative disease either alone or in combination with other therapies by administering to the cell a negative regulator of the IAP-dependent or anti-apoptotic pathway. The negative regulator may be, but is not limited to, an IAP ring zinc finger, and an IAP polypeptide that includes a ring zinc finger and lacks at least one BIR domain. Alternatively, apoptosis may be induced in the cell by administering an oligonucleotide encoding an IAP antisense RNA molecule administered directly or via gene therapy (see U.S. Pat. No. 5,576,208 for general parameters that may be applicable in the selection of IAP antisense RNAs).

The the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes oligomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages as well as oligomers having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

Specific examples of some preferred oligonucleotides envisioned for this invention may contain phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are those with CH_2 –NH—O—CH_2, CH_2—N(CH_3)—O—CH_2, CH_2—O—N(CH_3)—CH_2, CH_2—N(CH_3)—N(CH_3)—CH_2 and O—N(CH_3)—CH_2—CH_2 backbones (where phosphodiester is O—P—O—CH_2). Also preferred are oligonucleotides having morpholino backbone structures (Summerton, J.E. and Weller, D.D., U.S. Pat. No: 5,034,506). In other preferred embodiments, such as the

protein-nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide may be replaced with a polyamide backbone, the bases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (P.E. Nielsen, M. Egholm, R.H. Berg, O Buchardt, Science 199, 254, 1497). Other preferred oligonucleotides may contain alkyl and halogen-substituted sugar moieties comprising one of the following at the 2' position: OH, SH, SCH_3, F, OCN, $O(CH_2)_nNH_2$ or $O(CH_2)_n$ CH_3, where n is from 1 to about 10; C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3; OCF_3; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; $SOCH_3$; SO_2CH_3; ONO_2; NO_2; N_3; NH_2; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a conjugate; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group. Other preferred embodiments may include at least one modified base form. Some specific examples of such modified bases include 2-(amino)adenine, 2-(methylamino)adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalklyamino)adenine, or other heterosubstituted alkyladenines.

In yet another method, the negative regulator may be a purified antibody, or a fragment thereof, that binds specifically to an IAP polypeptide. For example, in one preferred embodiment, the antibody may bind to an approximately 26 kDa cleavage product of an IAP polypeptide that includes at least one BIR domain but lacks a ring zinc finger domain.

In two additional aspects, the invention features a transgenic animal and methods of using the mammal for detection of anti-cancer therapeutics. Preferably the mammal overexpresses an IAP polypeptide and/or expresses an IAP antisense RNA or IAP fragment. In one embodiment, the animal also has a genetic predisposition to cancer or

has cancer cells under conditions that provide for proliferation absent the transgenic construct encoding either the antisense RNA or fragment.

"Protein" or "polypeptide" or "polypeptide fragment" means any chain of more than two amino acids, regardless of post-translational modification (e.g., glycosylation or phosphorylation), constituting all or part of a naturally-occurring polypeptide or peptide, or constituting a non-naturally occurring polypeptide or peptide.

"Apoptosis" means the process of cell death wherein a dying cell displays a set of well-characterized biochemical hallmarks that include cell membrane blebbing, cell soma shrinkage, chromatin condensation, and DNA laddering. Cells that die by apoptosis include neurons (e.g., during the course of neurodegenerative diseases such as stroke, Parkinson's disease, and Alzheimer's disease), cardiomyocytes (e.g., after myocardial infarction or over the course of congestive heart failure), and cancer cells (e.g., after exposure to radiation or chemotherapeutic agents). Environmental stress (e.g., hypoxic stress) that is not alleviated may cause a cell to enter the early phase of the apoptotic pathway, which is reversible (i.e., cells at the early stage of the apoptotic pathway can be rescued). At a later phase of apoptosis (the commitment phase), cells cannot be rescued, and, as a result, are committed to die.

Proteins and compounds known to stimulate and inhibit apoptosis in a diverse variety of cells are well known in the art. For example, intracellular expression and activation of the caspase (ICE) family induces or stimulates apoptotic cell death, whereas expression of the IAPs or some members of the Bcl-2 family inhibits apoptotic cell death. In addition, there are survival factors that inhibit cell death in specific cell types. For example, neurotrophic factors, such as NGF inhibit neuronal apoptosis.

In some situations it may be desirable to artificially stimulate or inhibit apoptotic cell death by gene therapy or by a compound that mimics a gene therapeutic effect. For example, a cell that is susceptible to apoptosis induced by disease or environmental stress

may be made more resistant to apoptosis by introducing an expression vector encoding an anti-apoptotic protein (such as an IAP, a Bcl-2 family member, or a neurotrophin) into the cell. Conversely, a cancer cell may be made less resistant to apoptosis by introducing into it an expression vector encoding a pro-apoptotic protein (such as a caspase) or by introducing into it an antisense nucleic acid, for example, an IAP antisense nucleic acid, regardless of its length. In addition, placement of the encoded protein of interest under the translational regulation of a XIAP IRES ensures that copious quantities of the protein are produced, especially under cellular conditions during which most protein translation (i.e., cap-dependent protein translation) is down-regulated, e.g., when a cell is under environmental stress, and when a cell is at a threshold for entering the apoptotic pathway.

By "IAP gene" is meant a gene encoding a polypeptide having at least one BIR domain and a ring zinc finger domain that is capable of modulating (inhibiting or enhancing) apoptosis in a cell or tissue when provided by other intracellular or extracellular delivery methods (see, e.g., U.S. Patent No. 5,919,912, U.S.S.N. 08/576,965, and WO 97/06255). In preferred embodiments, the IAP gene is a gene having about 50% or greater nucleotide sequence identity to at least one of the IAP amino acid encoding sequences of Figs. 1 through 6, or portions thereof. Preferably the region of sequence over which identity is measured is a region encoding at least one BIR domain and a ring zinc finger domain. Mammalian IAP genes include nucleotide sequences isolated from any mammalian source. Preferably the mammal is a human.

The term "IAP gene" is meant to encompass any member of the family of genes that encode inhibitors of apoptosis. An IAP gene may encode a polypeptide that has at least 20%, preferably at least 30%, and most preferably at least 50% amino acid sequence identity with at least one of the conserved regions of one of the IAP members described herein (i.e., either the BIR or ring zinc finger domains from human or murine XIAP, HIAP1, and HIAP2). Representative members of the IAP gene family include, without limitation, the human and murine XIAP, HIAP1, and HIAP2 genes.

By "IAP protein" or "IAP polypeptide" is meant a polypeptide, or fragment thereof, encoded by an IAP gene.

By "BIR domain" is meant a domain having the amino acid sequence of the consensus sequence: Xaal-Xaal-Xaal-Arg-Leu-Xaal-Thr-Phe-Xaal-Xaal-Trp-Pro-Xaa2-Xaal-Xaal-Xaa2-Xaa2-Xaal-Xaal-Xaal-Xaal-Leu-Ala-Xaal-Ala-Gly-Phe-Tyr-Tyr-Xaal-Gly-Xaal-Xaal-Asp-Xaal-Val-Xaal-Cys-Phe-Xaal-Cys-Xaal-Xaal- Xaal-Xaal-Xaal-Xaal-Trp-Xaal-Xaal-Xaal-Asp-Xaal-Xaal-Xaal- Xaal-Xaal-His-Xaal-Xaal-Xaal-Xaal-Pro-Xaal-Cys-Xaal-Phe-Val, wherein Xaal is any amino acid and Xaa2 is any amino acid or is absent (SEQ ID NO: 216). Preferably the sequence is substantially identical to one of the BIR domain sequences provided for XIAP, HIAP1, or HIAP2 herein.

By "ring zinc finger" or "RZF" is meant a domain having the amino acid sequence of the consensus sequence: Glu-Xaal-Xaal-Xaal-Xaal-Xaal-Xaal-Xaa2-Xaal-Xaal-Xaal-Cys- Lys-Xaa3-Cys-Met-Xaal-Xaal-Xaal-Xaal-Xaal-Xaa3-Xaal-Phe-Xaal-Pro-Cys-Gly-His-Xaal-Xaal-Xaal-Cys-Xaal-Xaal-Cys-Ala- Xaal-Xaal-Xaal-Xaal-Xaal-Cys-Pro-Xaal-Cys, wherein Xaal is any amino acid, Xaa2 is Glu or Asp, and Xaa3 is Val or Ile (SEQ ID NO: 217).

Preferably the sequence is substantially identical to the RZF domains provided in U.S. Patent No. 6,133,437, incorporated herein by reference, for the human or murine XIAP, HIAP1, or HIAP2.

By "enhancing apoptosis" is meant increasing the number of cells that apoptose in a given cell population. Preferably the cell population is selected from a group including ovarian cancer cells, breast cancer cells, pancreatic cancer cells, T cells, neuronal cells, fibroblasts, or any other cell line known to proliferate in a laboratory setting. It will be appreciated that the degree of apoptosis enhancement provided by an apoptosis-enhancing compound in a given assay will vary, but that one skilled in the art can determine the statistically significant change in the level of apoptosis that identifies a compound that enhances apoptosis otherwise limited by an IAP. Preferably "enhancing

apoptosis" means that the increase in the number of cells undergoing apoptosis is at least 25%, more preferably the increase is 50%, and most preferably the increase is at least one-fold. Preferably the sample monitored is a sample of cells that normally undergo insufficient apoptosis (i.e., cancer cells). Methods for detecting a changes in the level of apoptosis (i.e., enhancement or reduction) are described herein.

By "proliferative disease" is meant a disease that is caused by or results in inappropriately high levels of cell division, inappropriately low levels of apoptosis, or both. For example, cancers such as lymphoma, leukemia, melanoma, ovarian cancer, breast cancer, pancreatic cancer, and lung cancer are all examples of proliferative disease.

By "IAP biological activity" is meant any activity known to be caused *in vivo* or *in vitro* by an IAP polypeptide.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) an IAP polypeptide.

By "transgene" is meant any piece of DNA that is inserted by artifice into a cell, and becomes part of the genome of the organism that develops from that cell. Such a transgene may include a gene that is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell that includes a DNA sequence that is inserted by artifice into a cell and becomes part of the genome of the organism that develops from that cell. As used herein, the transgenic organisms are generally transgenic mammals (e.g., rodents, such as rats or mice) and the DNA (transgene) is inserted by artifice into the nuclear genome.

By "transformation" is meant any method for introducing foreign molecules, for example, an antisense nucleic acid, into a cell. Lipofection, calcium phosphate precipitation, retroviral delivery, electroporation, biolistic transformation, and penetratin

are just a few of the teachings that may be used. For example, biolistic transformation is a method for introducing foreign molecules into a cell using velocity driven microprojectiles such as tungsten or gold particles. Such velocity-driven methods originate from pressure bursts that include, but are not limited to, helium-driven, air-driven, and gunpowder-driven techniques. Biolistic transformation may be applied to the transformation or transfection of a wide variety of cell types and intact tissues including, without limitation, intracellular organelles (e.g., and mitochondria and chloroplasts), bacteria, yeast, fungi, algae, animal tissue, and cultured cells. In another example, a foreign molecule (e.g., an antisense nucleic acid) can be translocated into a cell using the penetratin system as described, for example, by Prochiantz (Nature Biotechnology 16: 819-820, 1998; and Derossi et al. (Trends Cell Biol. 8: 84-87, 1998). In this system a penetratin peptide contains a transduction sequence that carries the peptide and a conjugated partner, for example, a phosphorothioate antisense nucleic acid (that is cross-linked through a disulfide bridge to the peptide) across the plasma membrane into the cell. The disulfide band is reduced inside the cell, releasing the partner.

By "antisense," as used herein in reference to nucleic acids, is meant a nucleic acid sequence, regardless of length, that is complementary to the coding strand or mRNA of an IAP gene. Preferably the antisense nucleic acid is capable of enhancing apoptosis when present in a cell that normally does not undergo sufficient apoptosis. Preferably the increase is at least 10%, relative to a control, more preferably 25%, and most preferably 1-fold or more. Preferably an IAP antisense nucleic acid comprises from about 8 to 30 nucleotides. An IAP antisense nucleic acid may also contain at least 40, 60, 85, 120, or more consecutive nucleotides that are complementary to a IAP mRNA or DNA, and may be as long as a full-length IAP gene or mRNA. The antisense nucleic acid may contain a modified backbone, for example, phosphorothioate, phosphorodithioate, or other modified backbones known in the art, or may contain non-natural internucleoside linkages.

By "ribozyme" is meant an RNA that has enzymatic activity, possessing site specificity and cleavage capability for a target RNA molecule. Ribozymes can be used to decrease expression of a polypeptide. Methods for using ribozymes to decrease polypeptide expression are described, for example, by Turner et al., (Adv. Exp. Med. Biol. 465:303-318, 2000) and Norris et al., (Adv. Exp. Med. Biol. 465:293-301, 2000).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%, preferably 85%, more preferably 90%, and most preferably 95% homology to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By "substantially pure polypeptide" is meant a polypeptide that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably the polypeptide is an IAP polypeptide that is at least 75%, more preferably at least 90%, and

most preferably at least 99%, by weight, pure. A substantially pure IAP polypeptide may be obtained, for example, by extraction from a natural source (e.g., a fibroblast, neuronal cell, or lymphocyte) by expression of a recombinant nucleic acid encoding an IAP polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants that accompany it in its natural state. Thus, a protein that is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

By "substantially pure DNA" is meant DNA that is free of the genes that, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or that exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding additional polypeptide sequence.

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence that directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., an IAP polypeptide, a recombinant protein or an RNA molecule).

By "reporter gene" is meant a gene whose expression may be assayed; such genes include, without limitation, glucuronidase (GUS), luciferase, chloramphenicol transacetylase (CAT), and Beta-galactosidase.

By "promoter" is meant a minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements that are sufficient to render promoter-dependent gene expression controllable for cell type-specific, tissue-specific or that are inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene.

By "operably linked" is meant that a gene and one or more regulatory sequences are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences.

By "conserved region" is meant any stretch of six or more contiguous amino acids exhibiting at least 30%, preferably 50%, and most preferably 70% amino acid sequence identity between two or more of the IAP family members, (e.g., between human HIAP1, HIAP2, and XIAP). Examples of preferred conserved regions include, without limitation, BIR domains and ring zinc finger domains.

By "detectably-labelled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule. Methods for detectably-labelling a molecule are well known in the art and include, without limitation, radioactive labelling (e.g., with an isotope such as ^{32}P or ^{35}S) and nonradioactive labelling (e.g., chemiluminescent labeling or fluorescein labelling).

By "purified antibody" is meant an antibody that is at least 60%, by weight, free from proteins and naturally occurring organic molecules with which it is naturally associated. Preferably the preparation is at least 75%, more preferably 90%, and most preferably at least 99%, by weight, antibody, e.g., an IAP-specific antibody. A purified antibody may be obtained, for example, by affinity chromatography using recombinantly-produced protein or conserved motif peptides and standard techniques.

By "specifically binds" is meant an antibody that recognizes and binds a protein but that does not substantially recognize and bind other molecules in a sample, e.g., a biological sample, that naturally includes protein.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Drawings

Fig. 1 is the human XIAP cDNA sequence (SEQ ID NO: 218) and the XIAP polypeptide sequence (SEQ ID NO: 219).

Fig. 2 is the human HIAP1 cDNA sequence (SEQ ID NO: 220) and the HIAP1 polypeptide sequence (SEQ ID NO: 221).

Fig. 3 is the human HIAP2 cDNA sequence (SEQ ID NO: 222) and the HIAP2 polypeptide sequence (SEQ ID NO: 223). The sequence absent in the HIAP2 variant is boxed.

Fig. 4 is the murine XIAP (also referred to as "miap-3") cDNA sequence (SEQ ID NO: 224) and encoded murine XIAP polypeptide sequence (SEQ ID NO: 225).

Fig. 5 is the murine HIAP1 (also referred to as "miap-1") cDNA sequence (SEQ ID NO: 226) and the encoded murine HIAP1 polypeptide sequence (SEQ ID NO: 227).

Fig. 6 is the murine HIAP2 (also referred to as "miap-2") cDNA sequence (SEQ ID NO: 228) and the encoded murine HIAP2 polypeptide (SEQ ID NO: 229).

Figs.7A through 7L are graphs showing the effect of antisense XIAP oligonucleotides on XIAP protein expression, relative to total protein (Figs. 7A, 7C, 7E, 7G, 7I, and 7K). Figs. 7B, 7D, 7F, 7H, 7J, and 7L are the total protein concentration values for each oligonucleotide transfection compared to mock transfection results that were used to normalize the above XIAP protein results.

Figs. 8A through 8C are graphs showing the effects of various antisense XIAP oligonucleotides, alone or in combination, on XIAP RNA (Fig. 8A) and protein (Fig. 8B). Fig. 8C is a graph of the total protein concentration values for each oligonucleotide transfection compared to mock transfection results, which were used to normalize the XIAP protein results shown in Fig. 8B.

Figs. 9A through 9D are graphs of the effects of antisense XIAP oligonucleotides on cell viability (Figs. 9A, 9C, and 9D), and chemosensitization in the presence of adriamycin (Fig. 9B).

Fig. 10 is a graph showing the effects of HIAP1 antisense oligonucleotides on HIAP1 RNA levels.

Fig. 11A is a densitometric scan of a Western blot showing the effects of HIAP1 antisense oligonucleotides on a cell's ability to block cycloheximide-induced upregulation of HIAP1 protein.

Fig. 11 is a graph showing the effects of HIAP1 antisense oligonucleotides on a cell's ability to block cycloheximide-induced upregulation of HIAP1 protein.

Fig. 12 is a graph showing the effects of HIAP1 antisense oligonucleotides on cytotoxicity, as measured by total protein.

Fig. 13 is a graph showing the validation of the sequence specificity for HIAP1 antisense oligonucleotide APO 2.

Fig. 14 is a graph showing the effect of HIAP1 antisense oligonucleotides on the chemosensitization of drug-resistant SF295 glioblastomas.

Fig. 15 is the human XIAP sequence containing a 5' UTR, the coding region, and a 3' UTR.

Fig. 16 is the human HIAP1 sequence containing a 5' UTR, the coding region, and a 3' UTR.

Detailed Description of the Invention

The present invention provides IAP antisense nucleic acid sequences that inhibit IAP biological activity, regardless of length, and methods for using them to induce apoptosis in a cell. The antisense nucleic acids of the present invention may also be used to form pharmaceutical compositions. The invention also features methods for enhancing apoptosis in a cell by administering a negative regulator of the IAP anti-apoptotic pathway other than antisense. Such negative regulators include, for example, an IAP polypeptide comprising a ring zinc finger having no more than two BIR domains, and a compound that prevents cleavage of an IAP polypeptide. Such negative regulators may also be used to form a pharmaceutical composition. These pharmaceutical compositions may be used to treat, ameliorate, improve, sustain, or prevent a proliferative disease, for example, cancer, or a symptom of a proliferative disease.

Administration

An IAP antisense nucleic acid, or other negative regulator of the IAP anti-apoptotic pathway may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer the compounds to patients suffering from a disease that is caused by excessive cell proliferation. Administration may begin before the patient is symptomatic. Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intraarterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, suppository, or oral administration. For example, therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for IAP modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

The formulations can be administered to human patients in therapeutically effective amounts (e.g., amounts which prevent, eliminate, or reduce a pathological condition) to provide therapy for a disease or condition. The preferred dosage of therapeutic agent to be administered is likely to depend on such variables as the type and extent of the disorder, the overall health status of the particular patient, the formulation of the compound excipients, and its route of administration.

If desired, treatment with an IAP antisense nucleic acid, IAP fragments, or other negative regulator of the anti-apoptotic pathway may be combined with more traditional therapies for the proliferative disease such as surgery or chemotherapy.

For any of the methods of application described above, the therapeutic antisense IAP nucleic acid or other negative regulator of the IAP anti-apoptotic pathway is preferably applied to the site of the needed apoptosis event (for example, by injection). However, it may also be applied to tissue in the vicinity of the predicted apoptosis event or to a blood vessel supplying the cells predicted to require enhanced apoptosis.

The dosage of an antisense IAP nucleic acid, or a negative regulator of the IAP anti-apoptotic pathway, for example, an IAP fragment, IAP mutant protein or an IAP antibody depends on a number of factors, including the size and health of the individual patient, but, generally, between 0.1 mg and 100 mg inclusive are administered per day to an adult in any pharmaceutically acceptable formulation. In addition, treatment by any IAP-modulating gene therapy approach may be combined with more traditional therapies.

Antisense Therapy

Anti-cancer therapy may be accomplished by direct administration of a therapeutic antisense IAP nucleic acid to a cell that is expected to require enhanced apoptosis. The antisense nucleic acid may be produced and isolated by any one of many standard techniques. Administration of IAP antisense nucleic acids to malignant cells can be carried out by any of the methods for direct nucleic acid administration, as described herein.

Retroviral vectors, adenoviral vectors, adeno-associated viral vectors, or other viral vectors with the appropriate tropism for cells likely requiring enhanced apoptosis (for example, breast cancer and ovarian cancer cells) may be used as a gene transfer delivery system for a therapeutic antisense IAP gene construct. Numerous vectors useful for this purpose are generally known (Miller, Human Gene Therapy 15-14, 1990; Friedman, Science 244:1275-1281, 1989; Eglitis and Anderson, BioTechniques 6:608-614, 1988; Tolstoshev and Anderson, Current Opinion in Biotechnology 1:55-61, 1990; Sharp, The Lancet 337:1277-1278, 1991; Cornetta et al., Nucleic Acid Research and Molecular Biology 36:311-322, 1987; Anderson, Science 226:401-409, 1984; Moen, Blood Cells 17:407-416, 1991; Miller et al., BioTechniques 7:980-990, 1989; Le Gal La Salle et al., Science 259:988-990, 1993; and Johnson, Chest 107:77S-83S, 1995).

Retroviral vectors are particularly well developed and have been used in clinical settings (Rosenberg et al., N. Engl. J. Med 323:370, 1990; Anderson et al., U.S. Patent

No. 5,399,346). Non-viral approaches may also be employed for the introduction of therapeutic DNA into cells otherwise predicted to undergo apoptosis. For example, IAPs may be introduced into a cell by lipofection (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413, 1987; Ono et al., Neurosci. Lett. 117:259, 1990; Brigham et al., Am. J. Med. Sci. 298:278, 1989; Staubinger et al., Meth. Enz. 101:512, 1983), the penetratin system (Allinquant et al., J. Cell Biol. 128:919-927, 1995; Prochiantz, Curr. Opin. Neurobiol. 6:629-634, 1996), asialorosonucoid-polylysine conjugation (Wu et al., J. Biol. Chem. 263:14621, 1988; Wu et al., J. Biol. Chem. 264:16985, 1989); or, less preferably microinjection under surgical conditions (Wolff et al., Science 247:1465, 1990).

In the therapeutic nucleic acid constructs described, nucleic acid expression can be directed from any suitable promoter (e.g., the human cytomegalovirus (CMV), simian virus 40 (SV40), or metallothionein promoters), and regulated by any appropriate mammalian regulatory element. For example, if desired, enhancers known to preferentially direct gene expression in ovarian cells, breast tissue, neural cells, T cells, or B cells may be used to direct expression. Enhancers include, without limitation, those that are characterized as tissue- or cell-specific in their expression. Alternatively, if a clone is used as a therapeutic construct, regulation may be mediated by the cognate regulatory sequences or, if desired, by regulatory sequences derived from a heterologous source, including any of the promoters or regulatory elements described above.

Therapeutic Products

For IAP related therapies one may employ the paradigms utilized for Bcl-2 and Ras antisense development, although accommodation of an IAP mutation is not required (in contrast to Ras antisense). Most useful are antisense constructs that enhance apoptosis at least 10%, preferably by enhancing degradation of the RNA in the nucleus.

Manipulation of cancer chemotherapeutic drug resistance using an antisense oligonucleotide and fragment approaches

We have documented that overexpression of the IAPs renders cell lines resistant to serum growth factor withdrawal, tumor necrosis factor alpha (TNF) and menadione exposure, all of which are treatments that normally induce apoptosis. Herein, we describe the extension of these studies to cancer cell lines using apoptotic triggers used in clinical situations, such as doxorubicin, adriamycin, and methotrexate. Our findings have led up to the design of antisense RNA therapeutics. Rapid screening of multiple cell lines for apoptotic response has been made feasible through the generation of a series of sense and antisense adenoviral IAP and expression vectors, as well as control lacZ viruses. One may now show enhanced drug resistance using the expression constructs. In addition, anti-sense adenovirus constructs may be developed and used to test reversal of the drug resistant phenotype of appropriate cell lines. We have designed a series of antisense oligonucleotides to various regions of each of the *iaps*. These oligonucleotides may be used to enhance drug sensitivity after testing in an assay system, i.e., with the adenoviral vectors system. Animal modeling of the effectiveness of antisense IAP oligonucleotides may also be employed as a step in testing and appropriate transgenic mammals for this are described in U.S. Patent No. 6,133,437, incorporated herein by reference, and are also generally available in the art.

Characterization of IAP Activity and Intracellular Localization Studies

The ability of IAPs to modulate apoptosis can be defined *in vitro* systems in which alterations of apoptosis can be detected. Mammalian expression constructs carrying IAP cDNAs, which are either full-length, truncated, or antisense constructs can be introduced into cell lines, such as CHO, NIH 3T3, HL60, Rat-1, or Jurkat cells. In addition, SF21 insect cells may be used, in which case the IAP gene is preferentially expressed using an insect heat shock promoter. Following transfection, apoptosis can be induced by

standard methods, which include serum withdrawal, or application of staurosporine, menadione (which induces apoptosis via free radial formation), or anti-Fas antibodies. As a control, cells are cultured under the same conditions as those induced to undergo apoptosis, but either not transfected, or transfected with a vector that lacks an IAP insert. The ability of each IAP related construct to inhibit or enhance apoptosis upon expression can be quantified by calculating the survival index of the cells, i.e., the ratio of surviving transfected cells to surviving control cells. These experiments can confirm the presence of apoptosis inhibiting activity and, as discussed below, can also be used to determine the functional region(s) of an IAP that may be employed to achieve enhancement of apoptosis. These assays may also be performed in combination with the application of additional compounds in order to identify compounds that enhance apoptosis via IAP expression.

Apoptosis Assays

Specific examples of apoptosis assays are provided in the following references. Assays for apoptosis in lymphocytes are disclosed by: Li et al., "Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein", Science 268:429-431, 1995; Gibellini et al., "Tat-expressing Jurkat cells show an increased resistance to different apoptotic stimuli, including acute human immunodeficiency virus-type 1 (HIV-1) infection", Br. J. Haematol. 89:24-33, 1995; Martin et al., "HIV-1 infection of human $CD4^+$ T cells *in vitro*. Differential induction of apoptosis in these cells." J. Immunol. 152:330-342, 1994; Terai et al., "Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1", J. Clin. Invest. 87:1710-1715, 1991; Dhein et al., "Autocrine T-cell suicide mediated by APO-l/(Fas/CD95)", Nature 373:438-441, 1995; Katsikis et al., "Fas antigen stimulation induces marked apoptosis of T lymphocytes in human immunodeficiency virus-infected individuals", J. Exp. Med. 1815:2029-2036, 1995;

Westendorp et al., "Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120", Nature 375:497, 1995; and DeRossi et al., Virology 198:234-44, 1994.

Assays for apoptosis in fibroblasts are disclosed by: Vossbeck et al., "Direct transforming activity of TGF-beta on rat fibroblasts", Int. J. Cancer 61:92-97, 1995; Goruppi et al., "Dissection of c-myc domains involved in S phase induction of NIH3T3 fibroblasts", Oncogene 9:1537-1544, 1994; Fernandez et al., "Differential sensitivity of normal and Ha-ras transformed C3H mouse embryo fibroblasts to tumor necrosis factor: induction of bcl-2, c-myc, and manganese superoxide dismutase in resistant cells", Oncogene 9:2009-2017, 1994; Harrington et al., "c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines", EMBO J., 13:3286-3295, 1994; and Itoh et al., "A novel protein domain required for apoptosis. Mutational analysis of human Fas antigen", J. Biol. Chem. 268:10932-10937, 1993.

Assays for apoptosis in neuronal cells are disclosed by: Melino et al., "Tissue transglutaminase and apoptosis: sense and antisense transfection studies with human neuroblastoma cells", Mol. Cell. Biol. 14:6584-6596, 1994; Rosenbaum et al., "Evidence for hypoxia-induced, programmed cell death of cultured neurons", Ann. Neurol. 36:864-870, 1994; Sato et al., "Neuronal differentiation of PC12 cells as a result of prevention of cell death by bcl-2", J. Neurobiol. 25:1227-1234, 1994; Ferrari et al., "N-acetylcysteine D- and L-stereoisomers prevents apoptotic death of neuronal cells", J. Neurosci. 1516:2857-2866, 1995; Talley et al., "Tumor necrosis factor alpha-induced apoptosis in human neuronal cells: protection by the antioxidant N-acetylcysteine and the genes bcl-2 and crmA", Mol. Cell Biol. 1585:2359-2366, 1995; and Walkinshaw et al., "Induction of apoptosis in catecholaminergic PC12 cells by L-DOPA. Implications for the treatment of Parkinson's disease", J. Clin. Invest. 95:2458-2464, 1995.

Assays for apoptosis in insect cells are disclosed by: Clem et al., "Prevention of apoptosis by a baculovirus gene during infection of insect cells", Science 254:1388-1390, 1991; Crook et al., "An apoptosis-inhibiting baculovirus gene with a zinc finger-like

motif", J. Virol. 67:2168-2174, 1993; Rabizadeh et al., "Expression of the baculovirus p35 gene inhibits mammalian neural cell death", J. Neurochem. 61:2318-2321, 1993; Birnbaum et al., "An apoptosis inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs", J. Virol. 68:2521-2528, 1994; and Clem et al., "Control of programmed cell death by the baculovirus genes p35 and IAP", Mol. Cell. Biol. 14:5212-5222, 1994.

The following examples are to illustrate the invention. They are not meant to limit the invention in any way.

Example 1: Testing of antisense oligonucleotides

1. Complete panel of adenovirus constructs. The panel may consist of approximately four types of recombinant virus. A) Sense orientation viruses for each of the IAP open reading frames. These viruses are designed to massively overexpress the recombinant protein in infected cells. XIAP, HIAP1, HIAP2, and NAIP. B) Antisense orientation viruses in which the viral promoter drives the synthesis of an mRNA of opposite polarity to the *iap* mRNA, thereby shutting off host cell synthesis of the targeted protein coding region. XIAP, HIAP1, HIAP2, and NAIP "antisense" constructs are used for production of such antisense IAPs. C) Sub-domain expression viruses. These constructs express only a partial IAP protein in infected cells. We have data indicating that deletion of the zinc finger of XIAP renders the protein more potent in protecting cell against apoptotic triggers. This data also indicates that expression of the zinc finger alone will indicate apoptosis by functioning as a dominant-negative repressor of XIAP function. XIAP- ZF and XIAP- BIR viruses are required. D) Control viruses. Functional analysis of the IAPs requires suitable positive and negative controls for comparison. Bcl-2 sense, Bcl-2 antisense, p53 sense, and Lac Z (negative control) viruses may be utilized.

2. Confirmation of recombinant adenovirus function. Verification of the sense adenovirus function involves infection of tissue culture cells and determination of protein expression levels. We have performed Western blot analysis of several of the recombinant adenoviruses, including NAIP, XIAP and XIAP- ZF. The remaining viruses may be readily assessed for protein expression using the polyclonal IAP antibodies. Functional analysis of the antisense viruses may be done at the RNA level using either Northern blots of total RNA harvested from infected tissue culture cells or ribonuclease protection assays. Western blot analysis of infected cells will be used to determine whether the expressed antisense RNA interferes with IAP expression in the host cell.

3. Documentation that IAP overexpression results in increased drug resistance. We have optimized cell death assays to allow high through-put of samples with minimal sample variation. Testing of the sense IAP adenoviruses for their ability to alter drug sensitivity of breast and pancreatic adenocarcinoma cell lines may be accomplished as follows. Cancer cell lines are infected with the recombinant viruses, cultured for 5 days, then subdivided into 24 well plates. Triplicate cell samples each receive increasing concentrations of the anti-cancer drug under investigation. Samples are harvested at 24, 48, and 72 hours post-exposure, and assayed for the number of viable cells in the well. The dose response curve is then compared to uninfected and control virus (both positive and negative) infected cells. One may document a dramatic increase in the relative resistance of the cancer cell lines when infected with the sense viruses, confirming our hypothesis that overexpression of the IAP proteins contributes to the anti-apoptotic phenotype of cancer cells. Initial experiments utilize the drugs doxorubicin and adriamycin.

4. Documentation that antisense IAP overexpression results in increased drug sensitivity. Having confirmed that IAP overexpression renders cancer cells more

resistant to chemotherapeutic drugs, one may examine whether the antisense adenoviruses render the same cells more sensitive. The effectiveness of antisense IAP viruses relative to antisense Bcl-2 virus will also be assessed as a crucial milestone.

5. Identification of antisense oligonucleotides. Concomitant to the adenovirus work, we have designed a series of antisense oligonucleotides to various regions of each of the IAPs. A generally accepted model of how antisense oligonucleotides function proposes that the formation of RNA/DNA duplexes in the nucleus activates cellular RnaseH enzymes which then enzymatically degrade the mRNA component of the hybrid. Virtually any region of the mRNA can be targeted, and therefore choosing an appropriate sequence to target is somewhat empirical.

6. Optimization of oligonucleotides. A secondary round of oligonucleotides may be made when effective target regions have been identified. These oligonucleotides target sequences in the immediate vicinity of the most active antisense oligonucleotides identified using methods such as those provided above. A second round of testing by Northern blot analysis may be required.

7. Testing antisense oligonucleotides *in vitro*. Following successful identification and optimization of targeting oligonucleotides, one may test these in the tissue culture model system using the optimal cell lines such as those described in the cancer survey described in U.S. Patent No. 6,133,437, incorporated herein by reference. Experimental procedures may parallel those used in the recombinant antisense adenovirus work. Negative control oligonucleotides with miss-match sequences are used to establish base line or non-specific effects. Assisted transfection of the oligonucleotides using cationic lipid carriers may be compared to unassisted transfection. Confirmation of the effectiveness of specific antisense oligonucleotides prompts synthesis of oligonucleotides with modified

phosphodiester linkages, such as phosphorothioate or methylimino substituted oligonucleotides. These may also be tested *in vitro*.

8. Animal modeling of antisense oligonucleotide therapies. Animal modeling of the effectiveness of the antisense IAP approach is described here. Cell lines are routinely assessed for their tumorigenic potential in "nude" mice, a hairless strain of mouse that is immunocompromised, and thus extremely susceptible to developing tumors. In the nude mouse assay, cancer cells are grown in tissue culture and then injected under the skin at multiple sites. The frequency with which these cells give rise to palpable tumors within a defined period of time provides an index of the tumorigenic potential of the cell line in the absence of interference by a functional immune system. Preliminary assessment of an antisense IAP therapeutic involves injection of cancer cells infected with the recombinant adenoviruses (sense, antisense, and control viruses) under the skin, and the tumorigenic index compared to that of untreated cells. One may also use this model to assess the effectiveness of systemic administration of antisense oligonucleotides in increasing the efficacy of anti-cancer drugs in the nude mouse model. Phosphorothioate or methylimino substituted oligonucleotides will be assessed at this stage. This type of antisense oligo has demonstrated enhanced cell permeability and slower clearance rates from the body in experimental animal models.

Example 2: Antisense oligonucleotide (ODN) selection

We selected 96 and 98, mostly non-overlapping, 19-mer antisense oligonucleotide (ODN) sequences for XIAP and HIAP1, respectively, based on the selection criteria listed below. In the case of XIAP, we selected 96 sequences (each being 19 nucleobases in length) (SEQ ID NOS: 1 through 96; Table 1) from a region approximately 1 kb upstream of the start codon to approximately 1 kb downstream of the stop codon of the cDNA sequence (Fig. 15). This blanketed approximately 50% of the coding region, and

immediate 5' and 3' UTR sequences (i.e., 96 19-mers span 1.8 kb of sequence, while the targeted region is approximately 3.5 kb in length, comprised of a coding region of 1.5 kb plus 1 kb at either side of UTR sequences).

Table 1. XIAP Antisense Oligonucleotides

SEQ ID NO:	Code	Position in XIAP Sequence	Antisense Oligonucleotide Sequence
1	A1	2	AAAATTCTAAGTACCTGCA
2	B1	21	TCTAGAGGGTGGCTCAGGA
3	C1	44	CAGATATATATGTAACACT
4	D1	78	TGAGAGCCCTTTTTTTGTT
5	E1	110	AGTATGAAATATTTCTGAT
6	F1	134	ATTGGTTCCAATGTGTTCT
7	G1	160	TTAGCAAAATATGTTTTAA
8	H1	185	TGAATTAATTTTTAATATC
9	A2	238	ATTCAAGGCATCAAAGTTG
10	B2	326	GTCAAATCATTAATTAGGA
11	C2	370	AATATGTAAACTGTGATGC
12	D2	411	GCAGAATAAAACTAATAAT
13	E2	430	GAAAGTAATATTTAAGCAG
14	F2	488	TTACCACATCATTCAAGTC
15	G2	508	CTAAATACTAGAGTTCGAC
16	H2	535	ACACGACCGCTAAGAAACA
17	A3	561	TATCCACTTATGACATAAA
18	B3	580	GTTATAGGAGCTAACAAAT
19	C3	607	AATGTGAAACACAAGCAAC
20	D3	638	ACATTATATTAGGAAATCC

SEQ ID NO:	Code	Position in XIAP Sequence	Antisense Oligonucleotide Sequence
21	E3	653	CTTGTCCACCTTTTCTAAA
22	F3	673	ATCTTCTCTTGAAAATAGG
23	G3	694	CCTTCAAAACTGTTAAAAG
24	H3	721	ATGTCTGCAGGTACACAAG
25	A4	759	ATCTATTAAACTCTTCTAC
26	B4	796	ACAGGACTACCACTTGGAA
27	C4	815	TGCCAGTGTTGATGCTGAA
28	D4	835	GTATAAAGAAACCCTGCTC
29	E4	856	CGCACGGTATCTCCTTCAC
30	F4	882	CTACAGCTGCATGACAACT
31	G4	907	GCTGAGTCTCCATATTGCC
32	H4	930	ATACTTTCCTGTGTCTTCC
33	A5	950	GATAAATCTGCAATTTGGG
34	B5	990	TTGTAGACTGCGTGGCACT
35	C5	1010	ACCATTCTGGATACCAGAA
36	D5	1029	AGTTTTCAACTTTGTACTG
37	E5	1059	ATGATCTCTGCTTCCCAGA
38	F5	1079	AGATGGCCTGTCTAAGGCA
39	G5	1100	AGTTCTCAAAAGATAGTCT
40	H5	1126	GTGTCTGATATATCTACAA
41	A6	1137	TCGGGTATATGGTGTCTGA
42	B6	1146	CAGGGTTCCTCGGGTATAT
43	C6	1165	GCTTCTTCACAATACATGG
44	D6	1192	GGCCAGTTCTGAAAGGACT

SEQ ID NO:	Code	Position in XIAP Sequence	Antisense Oligonucleotide Sequence
45	E6	1225	GCTAACTCTCTTGGGGTTA
46	F6	1246	GTGTAGTAGAGTCCAGCAC
47	G6	1273	AAGCACTGCACTTGGTCAC
48	H6	1294	TTCAGTTTTCCACCACAAC
49	A7	1316	ACGATCACAAGGTTCCCAA
50	B7	1337	TCGCCTGTGTTCTGACCAG
51	C7	1370	CCGGCCCAAAACAAAGAAG
52	D7	1393	GATTCACTTCGAATATTAA
53	E7	1413	TATCAGAACTCACAGCATC
54	F7	1441	GGAAGATTTGTTGAATTTG
55	G7	1462	TCTGCCATGGATGGATTTC
56	H7	1485	AAGTAAAGATCCGTGCTTC
57	A8	1506	CTGAGTATATCCATGTCCC
58	B8	1525	GCAAGCTGCTCCTTGTTAA
59	C8	1546	AAAGCATAAAATCCAGCTC
60	D8	1575	GAAAGCACTTTACTTTATC
61	H8	1610	ACTGGGCTTCCAATCAGTT
62	E8	1629	GTTGTTCCCAAGGGTCTTC
63	F8	1650	ACCCTGGATACCATTTAGC
64	G8	1669	TGTTCTAACAGATATTTGC
65	A9	1688	TATATATTCTTGTCCCTTC
66	B9	1696	AGTTAAATGAATATTGTTT
67	C9	1725	GACACTCCTCAAGTGAATG
68	D9	1745	TTTCTCAGTAGTTCTTACC

SEQ ID NO:	Code	Position in XIAP Sequence	Antisense Oligonucleotide Sequence
69	E9	1759	GTTAGTGATGGTGTTTTCT
70	F9	1782	AGATGGTATCATCAATTCT
71	G9	1801	TGTACCATAGGATTTTGGA
72	H9	1820	CCCCATTCGTATAGCTTCT
73	A10	1849	ATTATTTTCTTAATGTCCT
74	B10	1893	CAAGTGATTTATAGTTGCT
75	C10	1913	TAGATCTGCAACCAGAACC
76	D10	1945	CATCTTGCATACTGTCTTT
77	E10	1997	CCTTAGCTGCTCTTCAGTA
78	F10	2018	AAGCTTCTCCTCTTGCAGG
79	G10	2044	ATATTTCTATCCATACAGA
80	H10	2076	CTAGATGTCCACAAGGAAC
81	A11	2096	AGCACATTGTTTACAAGTG
82	B11	2123	AGCACATGGGACACTTGTC
83	C11	2144	CTTGAAAGTAATGACTGTG
84	D11	2182	CCTACTATAGAGTTAGATT
85	E11	2215	ATTCAATCAGGGTAATAAG
86	F11	2234	AAGTCAGTTCACATCACAC
87	G11	2375	CAGTAAAAAAAATGGATAA
88	H11	2428	TTCAGTTATAGTATGATGC
89	A12	2471	TACACTTAGAAATTAAATC
90	B12	2630	TCTCTATCTTTCCACCAGC
91	C12	2667	AGAATCCTAAAACACAACA
92	D12	2709	ATTCGCACAAGTACGTGTT

SEQ ID NO:	Code	Position in XIAP Sequence	Antisense Oligonucleotide Sequence
93	E12	2785	TGTCAGTACATGTTGGCTC
94	F12	2840	ACATAGTGTTTTGCCACTT
95	G12	2861	CTTTGATCTGGCTCAGACT
96	H12	2932	GAAACCACATTTAACAGTT

Note that the three most 5' and the three most 3' nucleobases may comprise DNA residues, or RNA residues, such as 2'-O methyl RNA residues. For example, the antisense oligonucleotide sequence of SEQ ID NO: 3 may be CAGATATATATGTAACACT or CAGATATATATGTAACACU.

A similar approach was taken for designing antisense oligonucleotides against HIAP1. Ninety-eight 19-mer sequences were chosen, with some of the latter sequences picked using less stringent criteria than the originally defined selection criteria (listed below), to increase the number of candidate sequences to study (SEQ ID NOs: 97 through 194; Table 2). Of these 98 sequences targeted to the HIAP1 sequence of Fig. 16, 15 (SEQ ID NOs: 97 through 104, 107, 113, 136, 156, 157, 181, and 193) were selected to evaluate the efficacy of decreasing HIAP1 expression. These 15 candidate sequences consisted of 4 sequences targeting the coding region (SEQ ID NOs: 136, 156, 157,and 181), 1 sequence targeting the 3' UTR (SEQ ID NO: 193), and 7 sequences targeting the 5'UTR (SEQ ID NOs: 100 through 104, 107, and 113; one of the 7 oligonucleotides overlapped the start codon), and 3 other oligonucleotides (SEQ ID NOs 97 through 99) that were designed to target an intronic segment of the 5'UTR (the value of which is discussed in Example 7). These above-described 15 HIAP1 antisense oligonucleotides were synthesized and tested.

Table 2. HIAP1 Antisense Oligonucleotides

SEQ ID NO:	Code	Position in HIAP1 Sequence	Antisense Oligonucleotide Sequence
97	APO 1	1152	TCATTTGAGCCTGGGAGGU
98	APO 2	1172	CGGAGGCTGAGGCAGGAGA
99	APO 3	1207	GGTGTGGTGGTACGCGCCT
100	APO 4	1664	ACCCATGCACAAAACTACC
101	APO 5	1865	AGAATGTGCCAGTAGGAGA
102	APO 6	2440	TCTCACAGACGTTGGGCTT
103	APO 7	2469	CCAGTGGTTTGCAAGCATG
104	APO 8	3695	GAAATTTAGTGGCCAGGAA
105		4013	AGAAATACACAATTGCACC
106		4032	TACTGATACATTTTAAGGA
107	APO 9	4057	TTCAACATGGAGATTCTAA
108		4076	ATTTCTATGCATTTAGAGT
109		4121	AATACTAGGCTGAAAAGCC
110		4142	GGCTTTGCTTTTATCAGTT
111		4165	TCTAGGGAGGTAGTTTTGT
112		4189	GGGAAGAAAAGGGACTAGC
113	APO 10	4212	GTTCATAATGAAATGAATG
114		4233	ATAAGAATATGCTGTTTTC
115		4265	TTCAAACGTGTTGGCGCTT
116		4283	ATGACAAGTCGTATTTCAG
117		4317	AAGTGGAATACGTAGACAT
118		4338	AGACAGGAACCCCAGCAGG
119		4357	CGAGCAAGACTCCTTTCTG
120		4376	AGTGTAATAGAAACCAGCA
121		4395	TGACCTTGTCATTCACACC
122		4426	TTATCCAGCATCAGGCCAC
123		4445	ACTGTCTCCTCTTTTCCAG
124		4464	TTTTATGCTTTTCAGTAGG
125		4489	ACGAATCTGCAGCTAGGAT
126		4517	CAAGTTGTTAACGGAATTT
127		4536	TAGGCTGAGAGGTAGCTTC
128		4555	GTTACTGAAGAAGGAAAAG
129		4574	GAATGAGTGTGTGGAATGT
130		4593	TGTTTTCTGTACCCGGAAG
131		4612	GAGCCACGGAAATATCCAC
132		4631	TGATGGAGAGTTTGAATAA
133		4656	GATTTGCTCTGGAGTTTAC
134		4670	GGCAGAAAATTCTTGATTT

SEQ ID NO:	Code	Position in HIAP1 Sequence	Antisense Oligonucleotide Sequence
135		4696	GGACAGGGGTAGGAACTTC
136	APO 11	4714	GCATTTTCGTTATTCATTG
137		4733	CTGAAAAGTAAGTAATCTG
138		4759	GGCGACAGAAAAGTCAATG
139		4812	CCACTCTGTCTCCAGGTCC
140		4831	CCACCACAGGCAAAGCAAG
141		4855	TTCGGTTCCCAATTGCTCA
142		4874	TTCTGACATAGCATTATCC
143		4893	TGGGAAAATGTCTCAGGTG
144		4907	TATAAATGGGCATTTGGGA
145		4926	TGTCTTGAAGCTGATTTTC
146		4945	GAAACTGTGTATCTTGAAG
147		4964	TGTCTGCATGCTCAGATTA
148		4988	GAATGTTTTAAAGCGGGCT
149		5007	CACTAGAGGGCCAGTTAAA
150		5040	CCGCACTTGCAAGCTGCTC
151		5070	CATCATCACTGTTACCCAC
152		5095	CCACCATCACAGCAAAAGC
153		5117	TCCAGATTCCCAACACCTG
154		5130	CCCATGGATCATCTCCAGA
155		5149	AACCACTTGGCATGTTGAA
156	APO 12	5168	CAAGTACTCACACCTTGGA
157	APO 13	5187	CCTGTCCTTTAATTCTTAT
158		5206	TGAACTTGACGGATGAACT
159		5225	TAGATGAGGGTAACTGGCT
160		5244	TGGATAGCAGCTGTTCAAG
161		5271	CATTTTCATCTCCTGGGCT
162		529	TGGATAATTGATGACTCTG
163		5309	GTCTTCTCCAGGTTCAAAA
164		5337	TATTCATCATGATTGCATC
165		5366	CATTTCCACGGCAGCATTA
166		5367	CCAGGCTTCTACTAAAGCC
167		5416	GCTAGGATTTTTCTCTGAA
168		5435	TCTATAATTCTCTCCAGTT
169		5454	ACACAAGATCATTGACTAG
170		5473	TCTGCATTGAGTAAGTCTA
171		5492	CTCTTCCCTTATTTCATCT
172		5515	TCCTCAGTTGCTCTTTCTC
173		5560	GCCATTCTATTCTTCCGGA

SEQ ID NO:	Code	Position in HIAP1 Sequence	Antisense Oligonucleotide Sequence
174		5579	AGTCAAATGTTGAAAAAGT
175		5598	CCAGGATTGGAATTACACA
176		5622	ATTCCGGCAGTTAGTAGAC
177		5646	TAACATCATGTTCTTGTTC
178		5675	GTCTGTGTCTTCTGTTTAA
179		5684	TTCTCTTGCTTGTAAAGAC
180		5703	CTAAAATCGTATCAATCAG
181	APO 14	5723	GGCTGCAATATTTCCTTTT
182		5742	GAGAGTTTCTGAATACAGT
183		5761	ACAGCTTCAGCTTCTTGCA
184		5780	AAATAAATGCTCATATAAC
185		5821	GAAACATCTTCTGTGGGAA
186		5841	GTTCTTCCACTGGTAGATC
187		5862	CTTCTTGTAGTCTCCGCAA
188		5890	TTGTCCATACACACTTTAC
189		6097	AACCAAATTAGGATAAAAG
190		6181	ATGTTCATATGGTTTAGAT
191		6306	TAAGTTTTACTTCACTTAC
192		6369	ATGTTCCCGGTATTAGTAC
193	APO 15	6432	GGGCTCAAGTAATTCTCTT
194		6455	GCCCAGGATGGATTCAAAC

Oligonucleotide selection criteria

The computer program OLIGO (previously distributed by National Biosciences Inc.) was used to define suitable antisense oligonucleotides based on the following criteria: 1) no more than 75% GC content, and no more than 75% AT content; 2) preferably no oligonucleotide with 4 or more consecutive G residues (due to reported toxic effects, although one was chosen as a toxicity control); 3) no oligonucleotides with the ability to form stable dimers or hairpin structures; and 4) sequences around the translation start site are a preferred region. In addition, accessible regions of the mRNA were predicted with the help of the RNA secondary structure folding program mfold by M. Zuker. Sub-optimal folds with a free energy value within 5% of the predicted most

stable fold of the mRNA were predicted using a window of 200 bases within which a residue can find a complimentary base to form a base pair bond. Open regions that did not form a base pair were summed together with each suboptimal fold and areas that consistently were predicted as open were considered more accessible to the binding of antisense oligonucleotides. Additional oligonucleotides that only partially fulfilled some of the above selection criteria (1-4), were also chosen as possible candidates if they recognized a predicted open region of the target mRNA.

Example 3: Antisense oligonucleotide synthesis

The antisense oligonucleotides were synthesized by IDT (Integrated DNA Technologies, USA) as chimeric, second-generation oligonucleotides, consisting of a core of phosphodiester DNA residues flanked on either side by two 2'-O methyl RNA residues with a phosphorothioate linkage between the flanking RNA residues. The oligonucleotides were provided in a 96-well plate, as well as matching tubes, with a minimum of 12 ODs of oligo DNA, which provided ample material for transfections (greater than a hundred assays in the 96-well format) when the detection method is a sensitive method, such as TaqMan quantitative PCR, or an ELISA. Once the positive hits were identified (see below), the antisense oligonucleotides were re-synthesized with 3, instead of 2, flanking RNA residues to further increase stability/nuclease resistance. In addition, for validation purposes, appropriate controls (such as scrambled, 4-base mismatch, and reverse polarity oligonucleotides) were synthesized for some of the antisense targets that yielded the highest antisense activity.

Example 4: Screening assays and optimization of antisense oligonucleotide sequences

Our approach to identifying IAP antisense oligonucleotides was to screen the above-described antisense oligonucleotide libraries for specific decreases (knock-down)

of the RNA and protein for the specific IAP gene targeted. Any number of standard assays may be used to detect RNA and protein levels in cells that have been administered an IAP antisense nucleic acid. For example, RNA levels can be measured using standard Northern blot analysis or RT-PCR techniques. In addition, protein levels can be measured, for example, by standard Western blot analyses or immunoprecipitation techniques. Alternatively, cells administered an antisense IAP nucleic acid may be examined for cell viability, according to methods described for example, in U.S. Patent Nos. 5,919,912 or 6,133,437, or U.S.S.N. 08/576,956, incorporated herein by reference.

We used TaqMan quantitative PCR conditions (described below) to assay for changes in mRNA levels after antisense oligonucleotide treatment, as well as our ELISA method for XIAP and Western blotting (described below) for changes in HIAP1 protein levels, using a polyclonal anti-RIAP1 antibody (rat HIAP1 ortholog; AEgera Therapeutics, Inc.) in the latter case. Transfection conditions were optimized with LipofectAMINE PLUS (Life Technologies, Canada) on T24 bladder carcinoma cells, or lipofectin on SF-295 glioblastoma cells, using a fluorescein-tagged control sense oligo from XIAP spanning the start codon (mGmAG AAG ATG ACT GGT AAmC mA; SEQ ID NO: 195). The results were visualized and gauged by epi-fluorescence microscopy. In addition, in the case of T24 cells, transfections were further optimized based on the ability of a published antisense oligonucleotide to downregulate survivin expression (Li et al., Nat. Cell Biol. 1:461-466, 1999) (U/TGT GCT ATT CTG TGA AU/TU/T SEQ ID NO: 196). We optimized the transfection conditions based on the TaqMan results of survivin RNA knock-down detected with PCR primers and fluorescent probe, described in detail below. Optimal conditions for oligo uptake by the cells were found to be 940 nM oligonucleotide and 40 μL PLUS reagent and 0.8 μL LipofectAMINE in a total of 70 μL for 3 hours. We then applied these conditions to screen for XIAP protein knock-down using the oligo library against T24 cells.

HIAP1 knock-down was studied in SF-295 cells because these cells had easily detectable and discernable 70 kDa HIAP1 protein, while many cell lines do not express high levels of the protein, or are not distinguishable from the large amounts of the similarly sized 68 kDa HIAP2 protein. In fact, there are a number of published errors involving HIAP1 and HIAP2 in the literature because of naming errors in the databases, and because of the poor quality and high crossreactivity, of the various commercial antibodies to HIAP1/cIAP2. The best way to distinguish HIAP1 from HIAP2 is to perform an immunoprecipitation experiment with an IAP antibody (Aegera Therapeutics, Inc.), separate the proteins by 2-dimensional gel electrophoresis, and to then carry out mass spectroscopy analysis of the spots migrating in the 68 to 70 kDa range to verify the identity of the HIAP1 and HIAP2 bands, using standard methods known in the art. This method determines if HIAP1 and HIAP2 co-migrate at the 68 kDa position, and if the 70 kDa form of HIAP1 results from a splice variant or a post-translational modification.

Real–time PCR

RNA was extracted from cells lysed in RLT buffer (QIAGEN, Inc., Canada), and purified using QIAGEN RNeasy columns/kits. Real-time quantitative PCR was performed on a Perkin-Elmer ABI 7700 Prism PCR machine. RNA was reverse transcribed and amplified according to the TaqMan Universal PCR Master Mix protocol of PE Biosystems, using primers and probes designed to specifically recognize XIAP, HIAP1, survivin, or GAPDH. For human survivin, the forward primer was 5'-TCTGCT TCAAGGAGCTGGAA-3', the reverse primer was 5'-GAAAGGAAAGCGCAA CCG-3', and the probe was 5'-(FAM)AGCCAGATGACGACCCCATAGAGGAAC ATA(TAMRA)-3' (SEQ ID NOs: 197 through 199). For human HIAP1, the forward primer was 5'-TGGAGATGATCCATGGGTTCA-3', the reverse primer was 5'-GAA CTCCTGTCCTTTAATTCTTATCAAGT-3', and the probe was 5'-(FAM)CTCACA

CCTTGGAAACCACTTGGCATG(TAMRA)-3' (SEQ ID NOs: 200 through 202). For human XIAP, the forward primer was 5'-GGTGATAAAGTAAAGTGCTTTCAC TGT-3', the reverse primer was 5'-TCAGTAGTTCTTACCAGACACTCCTCAA-3', and the probe was 5'-(FAM)CAACATGCTAAATGGTATCCAGGGTGCAAAT ATC(TAMRA)-3' (SEQ ID NOs: 203 through 205). For human GAPDH, the forward primer was 5'-GAAGGTGAAGGTCGGAGTC-3', the reverse primer was 5'-GAAGAT GGTGATGGGATTC-3', and the probe was 5'-(JOE)CAAGCTTCCCGTTCTCAG CC(TAMRA)-3' (SEQ ID NOs: 206 through 208).

Relative quantitation of gene expression was performed as described in the PE Biosystems manual using GAPDH as an internal standard. The comparative Ct (cycle threshold) method was used for relative quantitation of IAP mRNA levels compared to GAPDH mRNA levels. Briefly, real-time fluorescence measurements were taken at each PCR cycle and the threshold cycle (Ct) value for each sample was calculated by determining the point at which fluorescence exceeded a threshold limit of 30 times the baseline standard deviation. The average baseline value and the baseline SD are calculated starting from the third cycle baseline value and stopping at the baseline value three cycles before the signal starts to exponentially rise. The PCR primers and/or probes for the specific IAPs were designed to span at least one exon-intron boundary separated by 1 or more kb of genomic DNA, to reduce the possibility of amplifying and detecting genomic DNA contamination. The specificity of the signal, and possible contamination from DNA, were verified by treating some RNA samples with either DNase or RNase, prior to performing the reverse transcription and PCR reaction steps.

XIAP ELISA and HIAP1 western immunoblots

A standard colorimetric XIAP ELISA assay was performed using an affinity-purified rabbit polyclonal antibody to XIAP (Aegera Therapeutics, Inc.) as a capture antibody, and was detected with a XIAP monoclonal antibody (MBL, Japan) and a

biotinylated anti-mouse Ig antibody and horseradish peroxidase-conjugated streptavidin and TMB substrate. Alternatively, a polyclonal XIAP or HIAP1 antibody may be used to measure XIAP or HIAP1 protein levels, respectively.

HIAP1 was detected on a western immunoblot using an affinity-purified anti-RIAP1 rabbit polyclonal antibody as a primary antibody and was detected by ECL (Amersham) on X-ray film with a secondary horseradish-peroxidase-conjugated anti-rabbit Ig antibody and a chemiluminescent substrate. The anti-RIAP1 polyclonal antibody is raised against a GST-fusion of the rat ortholog of HIAP1. This antibody cross-reacts with both human and murine HIAP1 and HIAP2.

Example 5: Antisense XIAP oligonucleotides decrease XIAP RNA and polypeptide expression

The XIAP synthetic library of 96 antisense oligonucleotides was first screened for decreases in XIAP protein levels. Specifically, T24 cells (1.5×10^4 cells/well) were seeded in wells of a 96-well plate on day 1, and were cultured in antibiotic-free McCoy's medium for 24 hours. On day 2, the cells were transfected with XIAP antisense oligonucleotides as described above (oligonucleotides are labeled according to their plated position, i.e., A1 to H12, and include 2 repeats, A13 and B13 that contain lyophilized DNA pellets that stuck to the sealing membrane). Briefly, the oligos were diluted in 10 µl/well of serum-free, antibiotic-free McCoy's medium and then PLUS reagent was added. LipofectAMINE was diluted in 10 µl/well of serum-free, antibiotic-free McCoy's medium, and both mixes were incubated for 15 minutes at room temperature. The mixes were then combined and incubated for 15 minutes at room temperature.

In the meantime, the complete medium was removed from the cells and 50 µl/well of serum-free, antibiotic-free medium was added to the cells. The transfection mixes were added to the well, and the cells were incubated for 3 hours. Then 30 µl/well of

serum-free, antibiotic-free medium and 100 µl/well of antibiotic-free complete medium, containing 2X fetal bovine serum were added to each well.

At day 3, XIAP RNA levels were measured using quantitative real-time PCR techniques, as described above. At day 4, XIAP protein levels were measured by ELISA (Figs. 7A, 7C, 7E, 7G, 7I, and 7K) , and total cellular protein was measured biochemically (Figs. 7B, 7D, 7F, 7H, 7J, and 7L; used to normalize the XIAP protein levels). The results were compared to a mock transfection sample (treated with the transfection agent but no oligonucleotide DNA was added, and then processed as for the other samples). Time course experiments determined that the optimal time for protein knock-down to be around 12 to 24 hours.

The library was also screened for decreases in RNA levels, using TaqMan-specific PCR primers and fluorescent probes at the appropriate optimal time, using the primers and probes described above. Time course experiments determined mRNA to be optimally decreased at 6 to 9 hours. These results agree well with the protein results.

The first screen (although performed at a sub-optimal time point when XIAP levels are returning to normal, possibly due to an outgrowth of non-transfected cells) identified 16 antisense oligonucleotides (ODNs C2, E2, E3, F3, C4, D4, E4, F4, G4, C5, D5, B6, F6, D7, D8, F8) out of the total 96 antisense oligonucleotides tested that showed some decrease in XIAP protein levels relative to total protein, compared to mock (no ODN) transfection levels (Fig. 7A, 7C, 7E, 7G, 7I, and 7K). Interestingly, total protein was decreased for each of these 16 ODNs, which indicates a toxic or cytostatic effect of these ODNs (Fig. 7B, 7D, 7F, 7H, 7J, 7L). Note that ODNs B9 and C9 showed a clear drop in total protein but no relative drop in XIAP protein levels. These 16 hits were then validated more rigorously at more optimal time points XIAP protein and RNA knock-down results at 12 hours after the start of transfection.

The 16 antisense ODNs that showed some decrease in relative XIAP protein levels compared to mock transfection, were re-tested alone or in combination, with one control

oligo (D2) included, for their ability to knock-down XIAP protein at a more optimal time point (12 hours) based on the above described time course studies (Fig. 8B). these ODNs were also examined for their ability to decrease XIAP mRNA levels at 12 hours, normalized against GAPDH levels, and compared to mock transfection. Total protein concentrations at 12 hours were also determined (Fig. 8C).

There was a good correlation between the ability of an antisense ODN to decrease XIAP protein levels (Fig. 8B) with its ability to decrease XIAP mRNA levels (Fig. 8A). In addition, there is no major loss of total protein at this early time point, and the decrease in XIAP mRNA and protein precede the decrease in total protein that is seen at later time points. The ODNs that showed greater than 50% loss of XIAP protein or mRNA levels alone, or in a combination of two ODNs added at a 0.5:0.5 ratio, were identified as the best ODNs and validated further. Of these 16 oligonucleotides, 10 of them (ODNs E2, E3, F3, E4, F4, G4, C5, B6, D7, F8) showed a consistent ability to decrease XIAP protein or RNA levels by more than 50%, depending on the transfection conditions used, or when used in combination, as for ODNs C5 and G4.

Interestingly, these 16 oligonucleotides that demonstrated antisense activity clustered in 4 different target regions of the XIAP mRNA, with adjacent ODNs showing some knock-down activity. No antisense activity was observed by oligonucleotides that target sequences between these regions or islands of sensitivity. Presumably, these regions represent open areas on the mRNA that are accessible to antisense ODNs inside the cell. Two antisense oligonucleotides,E3 and F3, target XIAP just upstream of the start codon in the intervening region between the IRES and the translation start site, and partially overlap the end of the IRES element. ODNs C2, D2, and E2 target a XIAP region upstream of the minimal IRES element, providing further evidence that the minimal IRES region is a highly structured region of RNA which is not readily accessible to antisense ODNs *in vivo*. All the other antisense ODN hits fall within the coding region, including a cluster of activity at positions 856-916 of the HIAP sequence of Fig.

15 (ODNs E4, F4, and G4) and smaller separate areas, as demonstrated by ODNs C5 and D5, for example.

Example 6: XIAP antisense oligonucleotides increase cytotoxicity and chemosensitization

We also investigated if XIAP antisense ODNs could chemosensitize the highly drug resistant T24 cells to traditional chemotherapeutic drugs, such as adriamycin or cisplatin. Antisense ODNs were chosen to represent some of the different XIAP target regions and were tested for their cytotoxic effects, alone or in combination with other ODNs or drugs. Five of the ten best XIAP antisense oligonucleotides were tested for their ability to kill or chemosensitize T24 bladder carcinoma cells, and were compared to the effects of three corresponding scrambled control ODNs.

T24 cells were transfected with XIAP antisense oligonucleotides, scrambled oligonucleotides, no oligonucleotides (mock transfected), or were left untreated. The cells were tested for viability 20 hours after transfection (with the exception of the untreated control) using the WST-1 tetrazolium dye assay in which WST-1 tetrazolium dye is reduced to a colored formazan product in metabolically active cells (Fig 9A). Alternatively, cell viability is tested using any one of the above described apoptosis methods.

The occurrence of cytoxicity induced by the antisense XIAP ODN E4 was examined by visually inspecting T24 cells that were left untreated, mock transfected, or transfected with E4 antisense ODNs, E4 scrambled ODNs, E4 reverse polarity, or E4 mismatched ODNs. Twenty hours after transfection, the cells were examined for morphology (Fig. 9D). Only the cell transfected with antisense E4 ODNs showed signs of toxicity.

To examine the effects of the oligonucleotides on the chemosensitization of the T24 cells to cisplatin or adriamycin, oligonucleotides were tested for their ability to

further kill T24 cells in the presence of a fixed dose of adriamycin (0.5 μg/ml). Cells were first transfected with the oligonucleotides, then adriamycin was added for another 20 hours. Viability was measured by WST-1 at the end of the 20 hour drug treatment (Fig. 9B). Values are shown as percentage viability compared to their ODN treatment alone results shown in Fig. 9C. Fig. 9C is essentially a repeat of Fig. 9A, but with the actual corresponding values used in calculating the results for the chemosensitization experiment in Fig. 9B.

All 5 oligonucleotides tested (ODNs F3, E4, G4, C5, D7, or the combinations of E4+C5, or G4+C5) killed the T24 cells, leaving only 10-15% surviving cells after 24 hours, as compared to the mock (no ODN) transfected cells, or to cells transfected with 3 corresponding scrambled controls to F3 (mCmAmGAGATTTCATTTAAmCmGmU; SEQ ID NO: 209), E4 (mCmUmACgCTCgCCATCgTmUmCmA; SEQ ID NO: 210) and C5 (mUmGmCCCAAGAATACTAGmUmCmA; SEQ ID NO: 211)(Figs. 9A and 9C). Therefore, the toxicity is sequence-specific to those ODNs that reduce XIAP levels, and not to a non-sequence specific toxicity due to ODNs of this chemistry in general, as three scrambled controls did not show any more toxicity compared to the mock transfected control. This cytotoxicity may result from the combined effect of XIAP protein knock-down (and the expected loss of anti-apoptotic protection afforded by XIAP) and the cytotoxicity of the transfection itself. Both mock (no ODN) and scrambled ODN transfections resulted in an approximately 40% decrease in survival as compared to untreated cells (Fig. 9A). This is not unexpected, as the opposite is true (i.e., overexpression of IAPs protect insect cells from cytofectin-mediated cell death, a liposomal transfection agent similar to the ones used in these studies (Jones et al., J. Biol. Chem. 275:22157-22165, 2000)

The addition of a fixed dose of adriamycin or cisplatin at the end of the 3 hour transfection period resulted in a further decrease in survival for some of the tested oligonucleotides, a further 40% drop in survival after 20 hours for ODNs F3, D7 and

G4+C5 combination (Fig. 9B), compared to their corresponding ODNs treated values (Fig. 9C). Note that the values in Fig. 9B (ODN plus drug) are compared to their corresponding ODN survival (ODN alone) in Fig. 9C, which is set a 100% for each ODN. Only the results for adriamycin chemosensitization are shown; however, similar results were obtained when the cells were chemosensitized with cisplatin. At the fixed doses used, the mock and scrambled control transfections did not show any increased loss of survival when either treated with adriamycin (Fig. 9B). Chemosensitization is only seen when XIAP levels are decreased by a specific antisense ODN.

Example 7: Antisense HIAP1 oligonucleotides decrease HIAP1 RNA and polypeptide expression

The smaller library of 15 HIAP1 antisense oligonucleotides was screened for protein knock-down by Western and for RNA knock-down by TaqMan, using the primers and probes described above, under two different conditions. Alternatively, HIAP1 RNA levels may be detected using standard Northern blot analyses or RT-PCR techniques. The antisense oligonucleotides were administered to cells under basal conditions or under cycloheximide-induction conditions (24 hour treatment with sub-toxic doses). We have discovered that cycloheximide (CHX) can lead to a 10- to 200-fold induction of HIAP1 mRNA depending on the cell line treated. This in turn leads to an increase in HIAP1 protein, as seen on a western blot (70 kDa band). We have also discovered that this effect of CHX is via two distinct mechanisms of action. First, CHX activates NFkB, a known transcriptional inducer of HIAP1, by blocking the *de novo* synthesis of a labile protein, IkB, which is an inhibitor of NFkB. This effect is mimicked by puromycin, another protein synthesis inhibitor, and by TNF-alpha, which induces a signaling cascade leading to the phosphorylation, ubiquination, and degradation of IkB. However, only CHX leads to a further stabilization of the HIAP1 mRNA, as seen by the decreased rate of disappearance of HIAP1 message in the presence of actinomycin D, to block *de novo*

transcription, and CHX, as opposed to actinomycin D and puromycin or TNF-alpha combined.

SF295 glioblastoma cells were transfected with lipofectin and ODN (scrambled survivin, no oligo or mock, antisense APO1 to APO15) or left untreated. RNA was isolated from the cells 6 hours after transfection and the level of HIAP1 mRNA was measured by quantitative PCR (TaqMan analysis), normalized for GAPDH mRNA, with the value for the scrambled survivin ODN transfection set as 1.0.

The results of this experiment, a compilation of three separate experiments, are shown in Fig. 10. The scrambled survivin ODN, the mock transfection, and untreated (non-transfected) cells, all showed similar HIAP1 mRNA levels. Of the 15 antisense ODNs, 7 oligonucleotides (ODNs APO 1, -2, -7, -8, -9, -12, -15) showed an almost 50% decrease when compared to mock transfection or survivin scrambled control (mUmAmA GCTGTTCTATGTGmUmUmC; SEQ ID NO: 212) ODN transfection (Fig. 10). Some of the ODNs led to an induction in HIAP1 mRNA, which may be a stress response to a non-specific toxic ODN. The antisense ODN, however, may still be effective at knocking down HIAP1 protein levels even if the message is increased if the ODN is able to interfere with the translation process.

The effect of HIAP1 antisense oligonucleotides on HIAP1 protein and mRNA expression was also examined in cells induced to express HIAP1. SF295 cells were transfected with ODNs, or were mock transfected. The transfected cells were then treated with 10 μg/ml cycloheximide for 24 hours to induce 70 kDa HIAP1 mRNA and protein. Protein levels were measured by western immunoblot analysis with an anti-RIAP1 polyclonal antibody, and normalized against actin protein in a re-probing of the same blots. Scans of the western blot results are shown in Fig. 11A. The densitometric scan results were plotted against the mock results (set at 100%) in Fig. 11B. A line is drawn at 50% to easily identify the most effective antisense ODNs. The

transfection process itself (e.g., mock or scrambled survivin) induces HIAP1 protein compared to the untreated sample as shown on the western immunoblot.

Of the 15 tested ODNs, 6 of them (APO 1, -2, -7, -8, -12, and -15) showed the strongest activity, or had significant activity in both the protein and mRNA assays, and did not cause a stress-induced increase in HIAP1 mRNA, such as that seen with ODNs APO 4, -6, -11, -13, -14 (Fig. 10), and by control ODNs to APO 2 (mismatch or reverse polarity, see text below and Figs.12 and 13). Note that APO 6 also showed evidence of toxicity as seen by the general decrease in total protein (Fig. 12).

To further investigate the efficacy of HIAP1 antisense oligonucleotides under cycloheximide induction conditions, changes in HIAP1 mRNA were measured by TaqMan real time PCR 6 hours after transfection with ODN APO 2, which targets an Alu repeat within an intron of HIAP1 and results in the greatest block of CHX-induced upregulation of HIAP1 mRNA and protein. Controls for this experiment were three ODNs for APO 2: one scrambled sequence (same base composition but random order, AAG GGC GGC GGA GTG AGA C; SEQ ID NO: 213), one reverse polarity (same base composition, same sequential order but in the opposite direction, AGAGGACGGAGT CGGAGGC; SEQ ID NO: 214), and one mismatch sequence (containing 4 base mismatches out of 19 bases, CGGAGCGTGAGGATGGAGA; SEQ ID NO: 214).

Transfection of the APO 2 antisense into cells resulted in a 50% decrease in mRNA compared to a scrambled survivin control and matched perfectly with the protein results, while the scrambled control for APO 2 (H1 sc apo 2 in Fig. 13) did not change HIAP1 mRNA levels at all (repeated twice here, and in two different experiments). However, the mismatch control ODN (H1 mm apo 2) and the reverse polarity control ODN (H1 RV apo 2) showed an induction of 6 to 7 fold in HIAP1 mRNA at 6 hours. These ODNs no longer targeted HIAP1, as expected, but may still target Alu repeats because of the degeneracy and repeat nature of these sequences. Therefore, it is possible that these two controls are toxic to the cell and cause a stress response that leads to the

induction of HIAP1. This effect may also occur with the antisense APO 2 ODN, but in this case, the APO 2 ODN also causes the degradation of the induced HIAP1 mRNA which results in a relative decrease of HIAP1 mRNA, compared to a scrambled survivin control, as well as decreasing the relative fold induction of HIAP1 protein after transfection and CHX treatment, compared to scrambled survivin control ODN.

The six optimal antisense HIAP1 ODNs include two very effective antisense ODNs against an intronic sequence (APO 1, and -2; with APO 2 demonstrating the best activity). These oligonucleotides have some interesting properties that could be of great use therapeutically for cancer or autoimmune disorders. The oligonucleotides against an intronic sequence would likely only target pre-mRNA (very short-lived target) and not the mature, processed form of HIAP1. Typically, introns are not targeted for antisense except when one wants to alter splicing by targeting the intron-exon boundaries or the branching point. These usually result in the skipping of an exon rather than RNase-mediated degradation of the message. Both mechanisms would likely be favorable for the enhancement of apoptosis, as the skipping would result in the loss of the exon encoding the first two important BIR domains of HIAP1. The APO-2 antisense oligo also targets an intron of survivin for 18 consecutive bases out of 19, but we did not see any loss of survivin protein; only HIAP1 was decreased after the oligo treatment, demonstrating the specificity of the HIAP1 antisense oligonucleotide. These antisense oligonucleotides hit Alu sequences in the HIAP1 intron and potentially in many other genes, and induce the cancer cells to die (see below), which may be as a result of down regulating HIAP1 and some other critical genes, and thus of therapeutic value if it is not too toxic to normal cells.

Cancer cells have reportedly more Alu-containing transcripts and may therefore be more sensitive to apoptosis induction with an Alu targeting antisense ODN. Furthermore, this killing effect of APO 1 and APO 2 ODNs may be due to the combined effect of both targeting Alu sequences and HIAP1 simultaneously. This dual effect would result in an

effective way to prevent the normal stress response of HIAP1 induction through the NFkB pathway, when the cell is exposed to certain toxic agents. This stress response is most likely part of the cancer cell's anti-apoptotic program. By blocking HIAP1 expression, we counter this anti-apoptotic stress response and precipitate the cancer cell's demise.

Example 8: HIAP1 antisense oligonucleotides increase cytotoxicity and chemosensitization

The effect of HIAP1 antisense oligonucleotides on the chemosentization of SF295 cells was also evaluated. Cells were transfected with one of 3 different antisense ODNs (APO 7, APO 15, and Scrambled APO 2 (control)). Twenty-four hours after tranfection with the ODNs, the cells were incubated with adriamycin for an additional 24 hours before assaying by for cell survival by assaying WST-1.

The WST-1 survival curves for SF295 cells transfected with the above-described HIAP1 ODNs and then treated with increasing concentrations of adriamycin are shown in Fig. 14. The two ODNs that resulted in a decrease in HIAP1 mRNA also showed a decrease in survival when treated with adriamycin compared to cells treated with an ODN which did not reduce HIAP1 mRNA levels. Therefore, reducing HIAP1 levels by antisense, or other means, can chemosensitize a glioblastoma cell line that is highly resistant to the cytotoxic action of many chemotherapeutic drugs.

Example 9: *In vivo* analyses of IAP antisense oligonucleotides

Antisense oligonucleotides that decrease expression of IAP in cell culture models can be tested in animals. For example, the antisense oligonucleotide can be tested in mice according to the method of Lopes de Menezes et al. (Clin. Cancer Res. 6: 2891-2902, 2000) or Klasa et al. (Clin. Cancer Res. 6: 2492-2500, 2000). Antisense and control ODNs are tested, for example, in sub-cutaneous human xenografts of breast

cancer, colon cancer, lung cancer, squamous cell carcinoma or prostate cancer in SCID mice. The antisense oligonucleotides are also tested in an orthotopic model for the prostate, as well as in a disseminated non-Hodgkin's lymphoma model. The mouse's tolerance to cisplatin, taxol, doxorubicin, and cyclophosphamide is known for each of these models.

In vivo testing of the antisense oligonucleotides involves 15 intraperitoneal injections (once a day, on days 3 through 7, 10 through 14, and 17 through 21) of naked ODN of (5 mg/kg), with or without a chemotherapeutic drug. Alternatively, liposomal type carriers for the ODNs may also be employed. Oligos are injected shortly after tumors cells have been seeded in the mouse, or when the tumor has established and grown to a size of 0.1-0.15 g. Tumor size is then monitored to determine if the ODN treatments or ODN plus drug treatments reduce the growth rate of the tumor, lead to regression, or have no effect at all. In another alternative, ODNs in liposomal formulation are injected directly into the tumors.

Example 10: Anti-IAP antibodies

In order to generate IAP-specific antibodies, an IAP coding sequence (e.g., amino acids 180-276) can be expressed as a C-terminal fusion with glutathione S-transferase (GST; Smith et al., Gene 67:31-40, 1988). The fusion protein can be purified on glutathione-Sepharose beads, eluted with glutathione, and cleaved with thrombin (at the engineered cleavage site), and purified to the degree required to successfully immunize rabbits. Primary immunizations can be carried out with Freund's complete adjuvant and subsequent immunizations performed with Freund's incomplete adjuvant. Antibody titres are monitored by Western blot and immunoprecipitation analyses using the thrombin-cleaved IAP fragment of the GST-IAP fusion protein. Immune sera are affinity-purified using CNBr-Sepharose-coupled IAP protein. Antiserum specificity is

determined using a panel of unrelated GST proteins (including GSTp53, Rb, HPV-16 E6, and E6-AP) and GST-trypsin (which was generated by PCR using known sequences).

As an alternate or adjunct immunogen to GST fusion proteins, peptides corresponding to relatively unique hydrophilic regions of IAP may be generated and coupled to keyhole limpet hemocyanin (KLH) through an introduced C-terminal lysine. Antiserum to each of these peptides is similarly affinity-purified on peptides conjugated to BSA, and specificity is tested by ELISA and Western blotting, using peptide conjugates, and by Western blotting and immunoprecipitation using IAP expressed as a GST fusion protein.

Alternatively, monoclonal antibodies may be prepared using the IAP proteins described above and standard hybridoma technology (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, New York, NY, 1981; Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1994). Once produced, monoclonal antibodies are also tested for specific IAP recognition by Western blot or immunoprecipitation analysis (by the methods described in Ausubel et al., *supra*).

Antibodies that specifically recognize IAPs or fragments of IAPs, such as those described in U.S. Patent No. 6,133,437, incorporated herein by reference, containing one or more BIR domains (but not a ring zinc finger domain), or that contain a ring zinc finger domain (but not a BIR domain) are considered useful in the invention. They may, for example, be used in an immunoassay to monitor IAP expression levels or to determine the subcellular location of an IAP or IAP fragment produced by a mammal. Antibodies that inhibit the 26 kDa IAP cleavage product described herein (which contains at least one BIR domain) may be especially useful in inducing apoptosis in cells undergoing undesirable proliferation.

Preferably antibodies of the invention are produced using IAP sequence that does not reside within highly conserved regions, and that appears likely to be antigenic, as analyzed by criteria such as those provided by the Peptide structure program (Genetics Computer Group Sequence Analysis Package, Program Manual for the GCG Package, Version 7, 1991) using the algorithm of Jameson and Wolf (CABIOS 4:181, 1988). Specifically, these regions, which are found between BIR1 and BIR2 of all IAPs, are: from amino acid 99 to amino acid 170 of HIAP1, from amino acid 123 to amino acid 184 of HIAP2, and from amino acid 116 to amino acid 133 of either XIAP or m-XIAP. These fragments can be generated by standard techniques, e.g., by the PCR, and cloned into the pGEX expression vector (Ausubel et al., *supra*). Fusion proteins are expressed in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel et al. (*supra*). In order to minimize the potential for obtaining antisera that is non-specific, or exhibits low-affinity binding to IAP, two or three fusions are generated for each protein, and each fusion is injected into at least two rabbits. Antisera are raised by injections in series, preferably including at least three booster injections.

Example 11: Comparison of cell survival following transfection with full length vs. partial IAP constructs

In order to investigate the mechanism whereby human IAPs, including XIAP, HIAP1, and HIAP2, afford protection against cell death, expression vectors were constructed that contained either: (1) full-length IAP cDNA (as described in U.S. Patent No. 6,133,437), (2) a portion of an IAP gene that encodes the BIR domains, but not the RZF, or (3) a portion of an IAP gene that encodes the RZF, but not the BIR domains. Human and murine XIAP cDNAs were tested by transient or stable expression in HeLa, Jurkat, and CHO cell lines. Following transfection, apoptosis was induced by serum withdrawal, application of menadione, or application of an anti-Fas antibody. Cell death was then assessed by trypan blue exclusion. As a control for transfection efficiency, the

cells were co-transfected with a Beta-gal expression construct. Typically, approximately 20% of the cells were successfully transfected.

When CHO cells were transiently transfected, constructs containing full-length human or mouse XIAP cDNAs conferred modest but definite protection against cell death. In contrast, the survival of CHO cells transfected with constructs encoding only the BIR domains (i.e., lacking the RZF domain) was markedly enhanced 72 hours after serum deprivation. Furthermore, a large percentage of cells expressing the BIR domains were still viable after 96 hours, at which time no viable cells remained in the control, i.e. non-transfected, cell cultures, and less than 5% of the cells transfected with the vector only, i.e., lacking a cDNA insert, remained viable. Deletion of any of the BIR domains results in the complete loss of apoptotic suppression, which is reflected by a decrease in the percentage of surviving CHO cells to control levels within 72 hours of serum withdrawal.

Stable pools of transfected CHO cells, which were maintained for several months under G418 selection, were induced to undergo apoptosis by exposure to 10 μM menadione for 2 hours. Among the CHO cells tested were those that were stably transfected with: (1) full-length murine XIAP cDNA (miap), (2) full-length XIAP cDNA (XIAP), (3) full-length bcl-2 cDNA (Bcl-2), (4) cDNA encoding the three BIR domains (but not the RZF) of murine XIAP (BIR), and (5) cDNA encoding the RZF (but not BIR domains) of m-XIAP (RZF). Cells that were non-transfected (CHO) or transfected with the vector only (pcDNA3), served as controls for this experiment. Following exposure to 10 μM menadione, the transfected cells were washed with phosphate buffered saline (PBS) and cultured for an additional 24 hours in menadione-free medium. Cell death was assessed, as described above, by trypan blue exclusion. Less than 10% of the non-transfected or vector-only transfected cells remained viable at the end of the 24 hour survival period. Cells expressing the RZF did not fare significantly better. However, expression of full-length murine XIAP, human XIAP, or bcl-2, and expression of the

BIR domains, enhanced cell survival. When the concentration of menadione was increased from 10 μM to 20 μM (with all other conditions of the experiment being the same as when 10 μM menadione was applied), the percentage of viable CHO cells that expressed the BIR domain cDNA construct was higher than the percentage of viable cells that expressed either full-length murine XIAP or bcl-2.

Example 12: Analysis of the subcellular location of expressed RZF and BIR domains

The assays of cell death described above indicate that the RZF acts as a negative regulator of the anti-apoptotic function of IAPs. One way in which the RZF, and possibly other IAP domains, may exert their regulatory influence is by altering the expression of genes, whose products function in the apoptotic pathway.

In order to determine whether the subcellular locations of expressed RZF and BIR domains are consistent with roles as nuclear regulatory factors, COS cells were transiently transfected with the following four constructs, and the expressed polypeptide was localized by immunofluorescent microscopy: (1) pcDNA3-6myc-xiap, which encodes all 497 amino acids of SEQ ID NO:219, (2) pcDNA3-6myc-m-xiap, which encodes all 497 amino acids of mouse XIAP (SEQ ID NO:225), (3) pcDNA3-6myc-mxiap-BIR, which encodes amino acids 1 to 341 of m-xiap (SEQ ID NO:225), and (4) pcDNA3-6myc-mxiap-RZF, which encodes amino acids 342-497 of murine xiap (SEQ ID NO:225). The cells were grown on multi-well tissue culture slides for 12 hours, and then fixed and permeabilized with methanol. The constructs used (here and in the cell death assays) were tagged with a human Myc epitope tag at the N-terminus. Therefore, a monoclonal anti-Myc antibody and a secondary goat anti-mouse antibody, which was conjugated to FITC, could be used to localize the expressed products in transiently transfected COS cells. Full-length XIAP and MIAP were located in the cytoplasm, with accentuated expression in the peri-nuclear zone. The same pattern of

localization was observed when the cells expressed a construct encoding the RZF domain (but not the BIR domains). However, cells expressing the BIR domains (without the RZF) exhibited, primarily, nuclear staining. The protein expressed by the BIR domain construct appeared to be in various stages of transfer to the nucleus.

Other Embodiments

All publications and patent applications mentioned in this specification, including U.S. Patent Nos. 5,919,912 and 6,133,437, and U.S.S.Ns. 08/576,956, are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

What is claimed is: